

Chapter 6

Identification of the Binding Domains of Nedd4 E3 Ubiquitin Ligase with Its Substrate Protein TMEPAI

Lei Jing, Xin Huo, Yufeng Li, Yuyin Li and Aipo Diao

Abstract To investigate which domains of Nedd4 are responsible for the interaction with its substrate protein TMEPAI, the plasmids encoding GST-tagged WW domains of Nedd4 and His-HA-tagged TMEPAI were constructed, and the fusion proteins were expressed in *E. coli* BL21-CodonPlus (DE3)-RIL cells and purified. GST pull-down experiment indicated that Nedd4 directly interacts with His-HA-TMEPAI, and the WW2 and WW3 domains of Nedd4 are responsible for the interaction with TMEPAI, which represents a general mechanism for the interaction between Nedd4 and its substrate protein.

Keywords Nedd4 · WW domain · TMEPAI · GST pull-down

6.1 Introduction

Ubiquitination is a common protein modification, which regulates protein degradation, as well as protein transport and a variety of metabolic pathways in the cells [1]. The process of ubiquitination involves the ubiquitin-activating enzyme E1, ubiquitin-conjugating enzyme E2, and ubiquitin-protein ligating enzymes E3. Nedd4 (neuronal precursor cell expressed developmentally down-regulated 4) belongs to the Nedd4 of HECT E3 ubiquitin ligase family including Nedd4, Nedd4-2, Smurf1, Smurf2, Itch, WWP1, WWP2, Nedl1, and Nedl2 [2, 3]. Nedd4 contains a C2 domain, four WW domains, and a HECT domain [4]. The C2 domain binds to phosphor-lipids. The WW domain is 35–40 amino acids long containing two conserved tryptophan (W) residues

L. Jing · X. Huo · Y. Li · Y. Li · A. Diao (✉)

Key Lab of Industrial Fermentation Microbiology of the Ministry of Education,
School of Biotechnology, Tianjin University of Science and Technology,
Tianjin 300457, China
e-mail: diaoaiipo@tust.edu.cn

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spaced 21 amino acids apart. The WW domains interact with a variety of proline-based motifs, and recognize proline-containing phosphor-serine/phosphor-threonine sequences on the substrate protein. The HECT domain is comprised of approximately 350 amino acids and is responsible for ubiquitin transfer from a conserved cysteine residue to a lysine residue in the substrate protein [5–7].

TMEPAI is a Nedd4-binding protein that is originally identified as a prostate abundant, highly androgen-induced protein, and the *TMEPAI* gene is mapped to chromosome 20q13 [8]. TMEPAI protein contains two PY motifs (PPPY and PPTY), which are necessary for binding to the WW domains of Nedd4 ubiquitin ligase [8, 9].

It has been reported that TMEPAI is a substrate of Nedd4 through the interaction with the WW domains, however, which WW domains interact with TMEPAI is still unclear. In this study, we investigate the binding affinity of the four WW domains of Nedd4 with its substrate protein TMEPAI [10].

6.2 Materials and Methods

6.2.1 Construction of Plasmid

Four DNA fragments encoding the WW domains of Nedd4 were amplified by PCR using the primers listed in Table 6.1, and the four fragments were digested with *Bam*HI and *Eco*RI and inserted into the pGEX-4T-2 (GE Healthcare) vector. The *TMEPAI* gene was amplified by PCR with the forward primer 5'-AT-GAGCCACTACAAGCTGTCTG-3' and reverse primer 5'-CTAGA-GAGGGTGTCCCTTTC-3', and the PCR product was purified and digested with *Bam*HI and *Eco*RI, and inserted into the *Bam*HI and *Eco*RI sites of the pET21b vector.

Table 6.1 Primers used for PCR experiments

Target gene	Primer sequence
Nedd4-WW1 domain	
Forward	5'-CAACAACAAGAACCTTCTCCTCTAC-3'
Reverse	5'-CTTAGCCATTCTCAGCATCTGTTAGG-3'
Nedd4-WW2 domain	
Forward	5'-CTTTTGCCTACTTCATCTGGATTAC-3'
Reverse	5'-TTAGGTCAGCTGACTGGTCTCCACTG-3'
Nedd4-WW3 domain	
Forward	5'-TCTGAAATTGAGCAAGGATTC-3'
Reverse	5'-TTATGTCTTTCCTCTCAGATGGGCTG-3'
Nedd4-WW4 domain	
Forward	5'-ACTTCCAATGATCTAGGGCCTTAC-3'
Reverse	5'-TTACTACTGCTGGTCCAGTTATTGC-3'

6.2.2 Protein Expression and Purification

Plasmids encoding GST-fusion proteins of Nedd4 were transformed into *E. coli* BL21-CodonPlus (DE3)-RIL (Stratagene). Cells were induced with 0.1 mM IPTG overnight at 16 °C, and lysed in lysis buffer (20 mM Tris–Cl pH 7.4, 100 mM NaCl, 2 mM DTT) containing protease inhibitors. The recombinant protein was purified on Glutathione–Sephadex beads (GE Healthcare). After sonication, the extracts were clarified by centrifugation. The recovered supernatants were allowed to bind to Glutathione Sephadex beads for 3 h at 4 °C. The matrix was washed three times with lysis buffer, then the beads were eluted with elution buffer (100 mM Tris–Cl pH 7.4, 100 mM NaCl, 20 mM reduced glutathione). His-HA-TMEPAI was expressed in *E. coli* BL21-CodonPlus (DE3)-RIL (Stratagene) cells that transformed with pET21b-His-HA-TMEPAI plasmid, and induced with 0.5 mM IPTG overnight at 16 °C. The His-HA-tagged protein was purified on Ni-Sephadex beads after lysis of the bacteria in Na–P lysis buffer (50 mM Na–P pH 8.0, 300 mM NaCl, 5 mM β -mercaptoethanol) plus protease inhibitors. After sonication, the extracts were clarified by centrifugation. The recovered supernatants were allowed to bind to Ni-Sephadex beads for 3 h at 4 °C. The matrix was washed three times with Na–P lysis buffer containing 50 mM imidazole, the beads were eluted with elution buffer (50 mM Na–P pH 8.0, 300 mM NaCl, 5 mM β -mercaptoethanol, and 250 mM imidazole).

6.2.3 GST Pull-Down Assays and Western Blot

For the GST pull-down assays, 5 μ g GST-fusion proteins were first immobilized on the Glutathione–Sephadex beads. The matrix was incubated for 2 h in rotation at 4 °C, then washed three times with cold GST pull-down buffer (20 mM Tris–Cl pH 7.4, 100 mM NaCl, 1 % Triton-100, 2 mM DTT). At the same time, 0.5 μ g protein of His-HA-TMEPAI was resuspended in the GST pull-down buffer and clarified by centrifugation at 14,000 rpm for 10 min. The supernatant was added into the GST-beads and incubated for 2 h in rotation at 4 °C, then washed three times with cold GST pull-down buffer, and washed once with 1 \times PBS. Finally, the beads were resuspended and boiled in 40 μ L 2 \times SDS loading buffer; after boiled for 5 min, the supernatants were collected and separated by 10 % SDS-PAGE, electroblotted onto a polyvinylidene difluoride membrane (Millipore) and analyzed by Western blot using anti-HA-tag antibody.

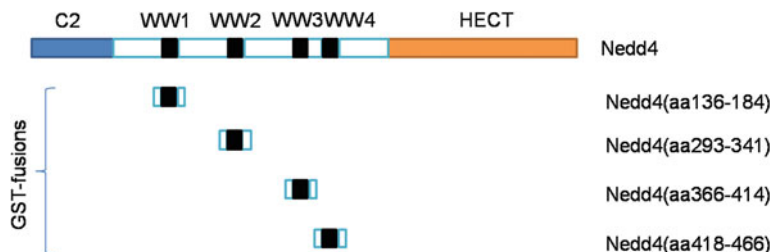


Fig. 6.1 The schematic structure of Nedd4. Amino acid positions of individual domains are denoted with corresponding numbers. C2, calcium-dependent lipid binding domain; WW, two conserved tryptophan (W) residues spaced 21 amino acids apart; HECT, homologous to E6 carboxyl terminus

6.3 Result

6.3.1 The Schematic Structure of Nedd4

Nedd4 (NP-006145) contains 900 amino acids, including a N-terminal C2 domain for membrane binding, a central region containing four WW domains for protein-protein interaction, and a C-terminal HECT domain for ubiquitin-protein ligation (Fig. 6.1).

6.3.2 Nedd4 Interacts with TMEPAI Directly

To verify that Nedd4 directly interacts with TMEPAI, GST-Nedd4, and His-HA-TMEPAI proteins were expressed in *E. coli* BL21-CodonPlus (DE3)-RIL and purified. GST pull-down experiment showed that GST-Nedd4, but not GST, pulled down purified His-HA-TMEPAI (Fig. 6.2), which indicates that GST-Nedd4 interacts with His-HA-TMEPAI directly.

6.3.3 Expression and Purification of the GST-Tagged WW Domains of Nedd4

To investigate which domains of Nedd4 are responsible for binding with TMEPAI, four plasmids encoding GST-tagged WW1, WW2, WW3, and WW4 domains of Nedd4 were constructed (Fig. 6.1). These four GST-fusion proteins were expressed and purified. The purified fusion proteins were subjected to SDS-PAGE and stained with Coomassie blue (Fig. 6.3).

Fig. 6.2 Direct interaction between Nedd4 and TMEPAI determined by the GST pull-down assay

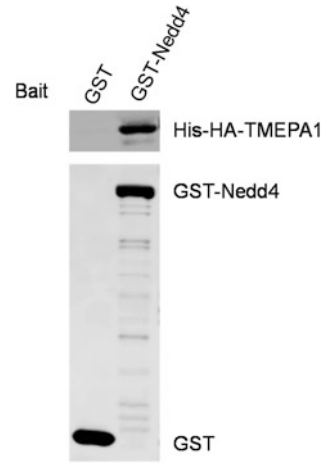
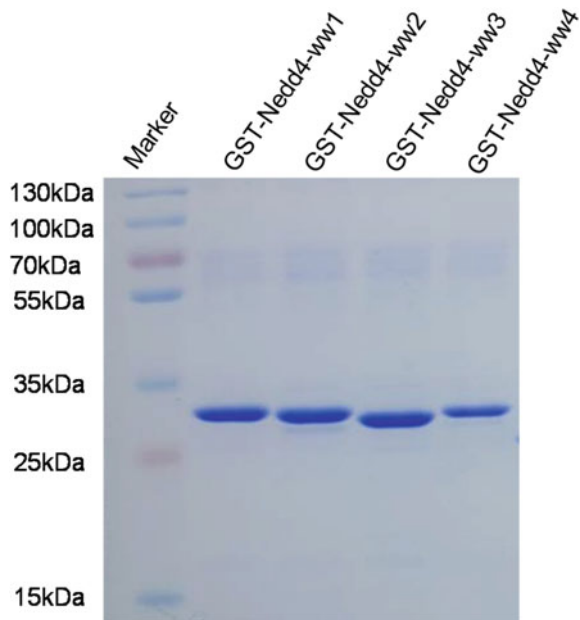


Fig. 6.3 GST-tagged WW domains of Nedd4 were expressed and purified using Glutathione–Sepharsose beads. The protein marker lane indicates the protein masses



6.3.4 *The WW2 and WW3 Domains of Nedd4 Interact with TMEPAI*

To measure the binding affinity of the Nedd4 WW domains with TMEPAI, the GST pull-down assay was performed. 5 μg GST or GST-WW domain fusion proteins were first bound to Glutathione–Sepharsose beads, then incubated with 0.5 μg

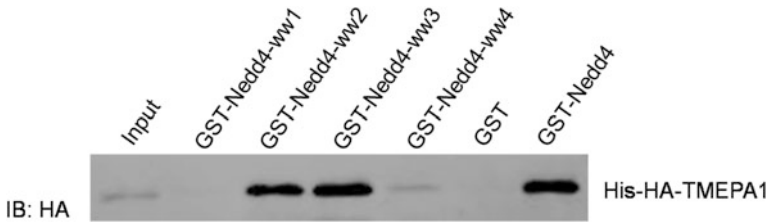


Fig. 6.4 GST pull-down assay to identify the interaction domain of Nedd4 with TMEPAI. GST or GST-WW domains fusion proteins coupled to the Glutathione–Sepharose beads were incubated with His-HA-TMEPAI for 2 h at 4°C and analyzed by Western blot. 5 % input represents 5 % of the material used for loading

His-HA-TMEPAI protein. GST pull-down assay showed that the WW2 and WW3 domain had a higher binding affinity with His-HA-TMEPAI, with a much less binding affinity for WW4 domain, but no binding affinity for WW1 domain (Fig. 6.4). These data indicate that the WW2 and WW3 domains of Nedd4 are mainly responsible for the interaction with TMEPAI.

6.4 Discussion

Previous studies have demonstrated that TMEPAI is a substrate of Nedd4 E3 ubiquitin ligase [5, 9, 11]. The mouse Nedd4 contains three WW domains and it has been shown that the WW2 domain plays an important role in protein interaction [12, 13]. In this study, we have identified that the WW2 and WW3 domains of Nedd4 play an important role in interacting with TMEPAI. This may represent a general mechanism for the interaction between Nedd4 and its substrates. However, we need to make a series of mutants of these proteins for further study.

Protein ubiquitination is a posttranslational modification in which a ubiquitin, or a multiubiquitin chain, is attached onto proteins by the formation of an isopeptide bond between the C-terminal Gly76 carboxyl group of ubiquitin and the 3-amino group of an internal Lys residue of the substrates, so as to control its stability or as a signal of protein transportation in the cells [14–17]. Given lacking of the specificity of proteasome inhibitors, the enzymatic nature, abundance, and specific substrate recognition properties of E3 ubiquitin ligase indicate that E3 might be used to as even more specific and effective therapeutic targets. Targeting individual E3 would selectively stabilize a particular or limit a number of protein substrates, thus possibly limiting untoward side effects. Hence, drugs targeting E3 promise a better therapeutic ratio than proteasome inhibitors, and the potential E3 inhibitors have a stronger chance to inhibit the active catalytic site.

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